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ORIGINAL ARTICLE

Astrocytes inhibit microglial surface expression of dendritic cell-related co-stimulatory molecules through a contact-mediated process

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Abstract

Murine microglia cultured in isolation were treated sequentially with granulocyte/monocyte colony-stimulating factor (GM-CSF) (5 days) and lipopolysaccharide (LPS) (2 days) to elicit a mature dendritic cell-like (DC-like) phenotype. Examined by flow cytometry microglia thus isolated show high surface expression of CD11c together with the co-stimulatory molecules CD40, CD80, and CD86 that are necessary for T-cell activation. In contrast, microglia co-cultured with astrocytes fail to achieve a mature DC-like phenotype. Contact with the astrocytic environment is necessary for the inhibition. Failure was not because of a more rapid degradation of protein. Bone marrow-derived cells, like microglia, were prevented by astrocytes from attaining a mature DC phenotype. Although GM-CSF pre-treatment substantially increases mRNA of co-stimulatory molecules and major histocompatibility complex (MHC) Class II in isolated microglia, co-cultured microglia await treatment with LPS to up-regulate them. In contrast, western blot and immunocytochemical analysis revealed that it is not a failure of transcription or translation, nor is it a more rapid degradation of mRNA that is responsible for the low surface expression; rather microglia co-cultured with astrocytes produce mRNA and protein but do not traffic the protein onto the cell surface.

Keywords: astrocytes, CD40, CD80, CD86, dendritic cells, microglia.

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Microglia are capable of expressing a mature dendritic cell-like (DC-like) phenotype. Although lacking (or having very low) MHC Class II expression *in vivo*, microglia can be stimulated in culture to express MHC Class II and co-stimulatory molecules CD40, CD80, and CD86 that are necessary for antigen presentation and T-cell activation (Ford *et al.* 1996; Kreutzberg 1996). We and others have shown that these molecules are up-regulated upon microglial isolation in culture (Satoh *et al.* 1995; Wei and Jonakait 1999). Granulocyte/monocyte colony-stimulating factor (GM-CSF) added into microglial cultures stimulates microglial proliferation (Lee *et al.* 1994; Liva *et al.* 1999) and up-regulates CD11c (a DC marker), MHC class II and co-stimulatory molecules (Fischer *et al.* 1993; Wei and Jonakait 1999; Re *et al.* 2002). These molecules can be further up-regulated by interferon-γ (IFN-γ) (DeGroot *et al.* 1991; Frei *et al.* 1994; Menendez *et al.* 1997; Aloisi *et al.* 1999; Nguyen and Benveniste 2000), lipopolysaccharide (LPS) (Menendez *et al.* 1997), CD40L (CD154) (Fischer and Reichmann 2001), and unmethylated CpG dinucleotides (Dalpke *et al.* 2002). As a result, isolated, stimulated microglia are effective T-cell activators *in vitro* (Cash and Rott 1994; Askew and Walker 1996; Matyszak *et al.* 1999; Aloisi *et al.* 2000; Fischer and Reichmann 2001; Re *et al.* 2002) suggesting the possibility that they can assume such a phenotype *in vivo* (Colton 2013).

Almost all of the experimental work done on the ability of microglia to assume a DC-like phenotype *in vitro* has involved their isolation from other glial populations and neurons. In routine culture preparations, mixed glial cultures are prepared and carried for 10–14 days followed by the *removal* of loosely adherent microglial populations. Thus

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Abbreviations used: BM, bone marrow; BM-DCs, bone marrow-derived DCs; ECM, extracellular matrix; FBS, fetal bovine serum; GM-CSF, granulocyte/monocyte colony-stimulating factor; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; TBST, tris-buffered saline containing 0.05% tween 20.

isolated, microglia attach firmly to tissue culture plastic and can be pharmacologically manipulated with relative ease. Studies on microglia thus isolated, like those cited above, have been useful in establishing microglial capabilities.

Microglia, however, do not exist in isolation; instead, they exist in a sea of other cell types, notably astrocytes. When cultured with astrocytes, microglia do not attach easily, but are detected as small cells hovering over the bed of astrocytes. To examine a possible role of astrocytes in regulating the DC-like phenotype, we have studied microglia both in their presence and absence. We find, indeed, that assumption of a DC-like phenotype is impeded when microglia are associated with astrocytes.

Materials and methods

Animals

C57BL/6 and B10.A mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in the Rutgers/Newark AAALAC-approved animal facility and all protocols were approved by the Rutgers Institutional Animal Care and Use Committee. Animals were mated on a regular basis to obtain neonatal pups for microglial cultures. In this study, 1–3-day-old pups were taken for glial cultures (see below).

Reagents

Recombinant mouse GM-CSF was purchased from Peprotech (Rocky Hill, NJ, USA). (LPS; 055:B5), trypsin, cytosine arabinoside (AraC), actinomycin D, and cycloheximide were from Sigma-Aldrich (St. Louis, MO, USA). Mouse Fc Block™ was from BD Biosciences (San Jose, CA, USA). Transwell™ inserts were from Corning (Lowell, MA, USA). Anti-CD11c and anti-CD4 magnetic beads were from Miltenyi Biotec (Sunnyvale, CA, USA).

Generation of mixed glial cultures

Mixed glial cultures were prepared as described previously from the cortices of neonatal (P1-P3) mouse pups (Jonakait *et al.* 1996, 2000; Ni *et al.* 2007). Cortices were cleared of meninges, minced, triturated, and plated into poly-lysine-coated 75 cm^2 flasks in medium containing Dulbecco's modified Eagle's medium/F12 (1 : 1), penicillin (25 U/mL), streptomycin (25 μg/mL), D-glucose (0.6%), and 10% heat-inactivated fetal bovine serum (Cell Generation, Ft. Collins, CO, USA). Medium was replaced on day 3 and half the medium exchanged every 3 days thereafter. Isolated microglial cultures were generated by shaking microglia off of 12–14-day-old mixed glial cultures on an orbital shaker (350 RPM × 20–30 min). Floating cells were collected and plated onto uncoated 75 cm^2 flasks and allowed to adhere to the substrate before being treated with GM-CSF and LPS. These constituted the 'isolated' culture condition. Microglia that remained in the mixed glial environment were considered 'co-cultured'.

Unless stated otherwise both mixed glial and isolated microglial cultures were treated with rmGM-CSF (25 ng/mL) for 5 days and LPS (50 ng/mL) for an additional 2 days to promote final maturation. Prior to analysis, co-cultured microglia were shaken off astrocytes (350 RPM × 20 min) so that analysis was of microglia alone, not of astrocytes.

Generation and purification of BM-DCs

Bone marrow (BM) cells were flushed with ice-cold RPMI medium from the femurs of 12–16-week-old B10.A mice. BM cells were cultured in 10 mL RPMI 1640 medium supplemented with 10% heat-inactivated Fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (25 U/mL), streptomycin (25 μg/mL), 50 μM beta-mercaptoethanol, and 15 ng/mL rmGM-CSF. After 3 days, floating cells were harvested, pelleted, and plated into 75 cm^2 flasks with or without enriched astrocytes (see below) in fresh RPMI medium containing GM-CSF (15 ng/ml). LPS (50 ng/mL) was added for 48 h on the fifth day of GM-CSF treatment. For experiments involving $CD11c^+$ purified BM cells, these were purified by CD11c immunomagnetic sorting using the AutoMACS™ system (Miltenyi Biotec). $CD11c^+$ cells treated with GM-CSF and LPS as described above. The purity of the sorted cells was determined by flow cytometric analysis (> 97% for $CD11c^+$ cells).

Generation of enriched astrocyte cultures

Enriched astrocyte cultures were prepared from mixed glial cultures from which microglia had been shaken off. Following the removal of loosely adherent microglia, astrocyte cultures were treated for 3 days with AraC (100 μM). They were passaged 2–3 times by light trypsinization (0.2% trypsin) followed by washing and re-plating into poly-lysine-coated flasks. Thus processed, they are > 95% pure as defined by staining of microglial contaminants with antibodies against CD11b.

Flow cytometric analysis

Microglia were harvested from mixed glia or from isolation culture and incubated for 30 min. at 4°C in buffer containing 2% FBS in phosphate-buffered saline (PBS) with PE-labeled hamster anti-mouse CD11c (7 μg/mL; BioLegend, San Diego, CA, USA). After 30 min, FITC-conjugated hamster anti-mouse CD40 (5 μg/mL; BD Biosciences) or FITC-conjugated CD80, CD86, or MHC Class II (10 μg/mL; BD Biosciences) were added for an additional 30 min. Non-specific binding was minimized by pre-incubation with FcBlock™ (BD Pharmingen, San Jose, CA) used according to manufacturer's instructions. Other cells were incubated with isotype controls, PE-labeled hamster anti-IgG (7 μg/mL; BioLegend), FITC-labeled hamster anti-IgM (5 μg/mL; BD Biosciences), FITC-labeled mouse $IgG_{2a}\kappa$, hamster $IgG_2\kappa$, or rat $IgG_{2a}\kappa$ (10 mg/mL; BD Biosciences). Isotype controls were prepared separately for isolated cells and co-cultured cells. Cells were then washed, pelleted, and re-suspended in the same buffer for flow cytometric analysis using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountainview, CA, USA). For experiments in which surface and total CD40 protein were to be detected, cells were initially stained with antibodies against CD11c and CD40. They were then fixed and permeabilized for 30 min (Fixation/Permeabilization Concentrate and Diluent, eBiosciences, San Diego, CA, USA), divided into two groups, and one group was re-stained with antibodies against CD40.

Because of the variability in the side-scatter [isolated and co-cultured cells show differences in their morphology (see below)], several controls were done to adjust for these differences. As isotype controls were used for all markers in both groups, isolated microglia and co-cultured microglia were compensated individually. Finally, unstained cells from each group were used in setting the initial gates. Thus, both groups were assessed individually. Even though bone marrow-derived DCs do not exhibit the same morphological

variability as do microglia, the same controls were used in analyzing these cells.

Data analysis was accomplished using CellQuest® software (BD Biosciences).

Quantitative real-time PCR

Total microglial RNA was prepared using Trizol™ RNA Isolation Reagent (Ambion, Life Technologies, Grand Island, NY, USA). cDNA was produced from 0.5 μg of RNA using random hexamer and MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instruction. The primers used for real-time PCR were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and were designed using Primer Express® (Applied Biosystem, Foster City, CA, USA). They are listed in Table 1. For QRT-PCR, cDNA was amplified using the SYBR Green PCR Master Mix (Applied Biosystem) according to manufacturer's instructions. PCR conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 15 s at 60°C to amplify. After amplification, an additional cycle consisting of 95°C and 60°C (each for 15 s) was used for a dissociation curve to verify that the signal is generated from a single target amplicon and not from primer dimers or contaminating DNA. Serially diluted cDNA of each sample was amplified to measure the efficiencies of PCR and to draw the standard curve for each sample to calculate relative concentration of target message. The PCR products and their dissociation curves were detected using the ABI Prism 7900HT Sequence Detection System (Applied Biosystem).

Rate of protein degradation

Isolated microglia or co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 16 h at which point cycloheximide (CHX, 10 μg/mL) was added to inhibit translation. At the various time points after the addition of CHX, cells were harvested and assessed for surface protein expression of CD11c and CD40 by flow cytometry. The percent of $CD11c^{+}CD40^{+}$ cells remaining at each time point was determined. Data were expressed as the logarithm of that percentage. Differences between the logs at time intervals 0–4, 4–8, 8–16, and 16–24 h were calculated for both co-cultured and isolated microglia. Those differences were compared with an ANOVA and a *post hoc* Student–Newman–Keuls test to obtain a measure of statistical significance.

Rate of mRNA degradation

Isolated microglia or co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 3 h at which point actinomycin D (5 μg/mL) was added to inhibit transcription. After 0.5, 1, 2, and 4 h, cells were harvested and assessed for mRNA by RT-PCR for CD40, CD80, and CD86. The percent of mRNA remaining at each time point was determined and that percentage was log transformed. Differences between the logs at time intervals 0–0.5, 0.5–1, 1–2, and 2–4 h were calculated for both co-cultured and isolated microglia. Those differences were compared with an ANOVA with a *post hoc* Student–Newman–Keuls test to obtain a measure of statistical significance.

Table 1 Primers used

CD40	5′ CTG CCC AGT CGG CTT CTT CTC 3′ F
	5′ CCT GTG TGA CAG GCT GAC AC 3′ R
CD80	5′ CTG GGA AAA ACC CCC AGA AG 3′ F
	5′ TGA CAA CGA TGA CGA CGA CTG 3′ R
CD86	5′ TTA CGG AAG CAC CCA TGA TG 3′ F
	5′ CGT CTC CAC GGA AAC AGC AT 3′ R
MHC Class II	5′ CAA CAC TCT GGT CTG CTC AGT GA 3′ F
	5′ TGT GTG GAT GAG ACC CCC A 3′ R

Western blotting

Microglia were harvested from mixed glia or from isolation culture at various time points after LPS treatment. Cells were lysed in buffer containing 10% glycerol, 1% NP40, 2.5 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 150 mM NaCl, and Protease Inhibitor Cocktail (1 : 100; Sigma, St Louis, MO, USA) in a 20 mM Tris buffer (pH 7.4). Protein concentration was measured using a Bio-Rad protein assay. Protein samples (30 μg each) were size-fractionated on a 15% sodium dodecyl sulfate–polyacrylamide gel, and transferred overnight onto nitrocellulose membranes. After blocking with 5% dry milk, the membranes were probed with antibodies against CD40 (HM40-3, 1 : 100; BD Pharmingen™). All antibodies were diluted in buffer containing 5% bovine serum albumin in 1× Tris-buffered saline containing 0.05% Tween 20 (TBST). After vigorous washing in TBST, membranes were probed with HRP-conjugated secondary antibodies (1 : 2500) in 5% milk in TBST. For HM40-3, the secondary antibody (1 : 1250) was in 5% bovine serum albumin in TBST. Bands were detected by enhanced chemiluminescence.

Immunocytochemistry

After 12–14 days, microglia were shaken from mixed glial cultures and seeded onto glass coverslips. Remaining mixed glia (in flasks) and the isolated microglia (on coverslips) were treated with GM-CSF (5 days) and LPS for various periods of time. For immunocytochemical detection of CD40, isolated microglia on coverslips were rinsed with PBS and fixed with 4% paraformaldehyde for 20 min. Microglia that were co-cultured with astrocytes were shaken off (350 RPM × 30 min) and seeded onto glass coverslips. They were allowed to settle and attach to the coverslips for 20 min prior to rinsing and fixation. Living cells were stained with ER Tracker™ (1 μM) or LysoTracker™ (1 μM) from Molecular Probes (Invitrogen Detection Technologies, Grand Island, NY, USA). They were then fixed with 4% paraformaldehyde for 5 min, blocked and permeabilized with 5% FBS, 0.1% triton in PBS for 20 min. This was followed by incubation with FITC-conjugated CD40 antibody (HM40-3; 1:750) for 45 min. For antibodies against EEA-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cells were fixed, blocked and permeabilized, and treated with the antibody (1 : 100) for 45–60 min at 37°C prior to inclusion of the CD40 antibody. Finally, the cells were mounted in ProLong® Gold anti-fade reagent (Invitrogen). After 24 h, cells were examined using a Zeiss LSM 510META confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA) and analyzed with ImageJ® software (rsb.info.nih.gov/ij/).

Results

Microglia cultured with astrocytes resist the assumption of a mature DC-like phenotype

Bone marrow-derived DCs and microglia require a priming signal (GM-CSF) as well as a secondary signal to achieve a

mature DC phenotype. In the case of microglia, these treatments have been performed routinely *in vitro* on microglia isolated from their more complex glial/neuronal environment. To determine whether treatment of microglia in the presence of astrocytes would prompt similar DC-like maturation, mouse microglia were cultured in the presence (Co-cultured) or absence (Isolated) of astrocytes with GM-CSF for 5 days. They then received LPS, a ligand at Toll-like receptor 4 (TLR4), for two additional days.

Flow cytometry indicated that a greater percentage of isolated microglia expressed the DC marker, CD11c ($73.4 \pm 4.2\%$ [isolated] vs. $48.9 \pm 6.5\%$ [co-cultured]; $n = 5$; $p = 0.019$). Moreover, the percentage of isolated microglia simultaneously expressing surface CD11c together with CD40, CD80, or CD86 was also significantly higher than co-cultured microglia (Fig. 1a and b). However, there was no significant difference in the percentage of microglia expressing CD11c and MHC Class II in the different environments (Fig. 1b).

While the percentages of cells expressing several DC markers was strikingly different, the mean fluorescence intensity (MFI), a measure of the number of molecules per cell, showed that only CD40 differed significantly between the two culture conditions (Fig. 1c).

To determine whether the lower levels of CD40 surface expression as reflected by the MFI were because of a more rapid degradation of translated protein in co-cultured microglia, cells were cultured with their usual GM-CSF/LPS protocol, but 16 h after LPS addition, cultures were treated with cycloheximide (CHX, 10 μg/mL) to halt protein translation (Fig. 1d). At various time points following these treatments, cells were harvested and assessed by flow cytometry for surface protein expression. The slopes of decay indicated that CD11c, CD40 and the simultaneous

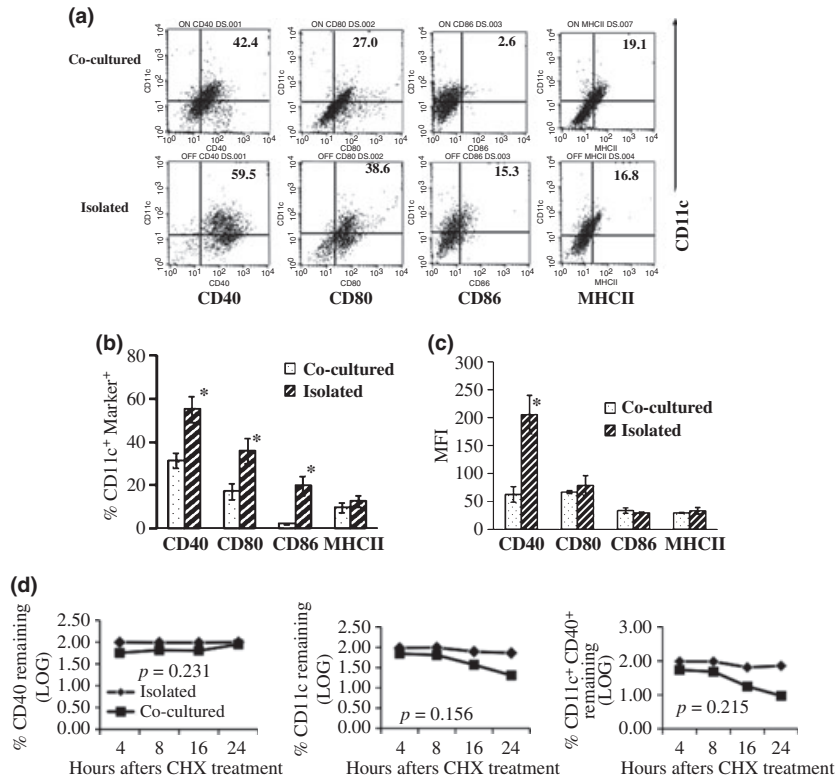

Fig. 1 Astrocytes inhibit microglial production of a mature DC phenotype. Microglia were cultured with granulocyte/monocyte colony-stimulating factor (GM-CSF) (25 ng/mL) in the presence (Co-cultured) or absence (Isolated) of astrocytes for 5 days. Lipopolysaccharide (LPS) (50 ng/mL) was then added for an additional 2 days. Co-stimulatory molecule surface expression was determined by flow cytometry (a and b). Gates were determined using isotype controls. Numbers in the upper right quadrants represent the percentage of $CD11c^+$ cells simultaneously positive for CD40, CD80, CD86, and MHC Class II, respectively. (b) Microglial surface expression of co-stimulatory molecules in isolation is significantly higher than those on microglia cultured with astrocytes. Data are expressed as the percentage of cells expressing both CD11c and another co-stimulatory molecule ± SEM over three individual experiments. Data from isolated and co-cultured microglia were compared by a Student's *t*-test. An asterisk indicates $p < 0.05$. (c) Only CD40 Mean Fluorescence Intensity (MFI) differs in the presence (Co-cultured) and absence (Isolated) of astrocytes. Data are expressed as the MFI ± SEM and compared by a Student's *t*-test. The asterisk indicates $p < 0.05$. (d) Protein degradation rate does not account for differences in surface expression of protein. Protein degradation rate was determined as described in *Materials and Methods*. *p*-values obtained are noted on the graphs. A representative experiment of three is shown.

expression of surface CD11c and CD40 were not significantly different between the two culture conditions, suggesting that the observed difference is not because of the rapid degradation of CD40 or CD11c on co-cultured microglia.

Repression of the DC-like phenotype requires contact with an astrocytic environment

To ensure that astrocytes and not other glial or neuronal components were responsible for the inhibition of the DC-like phenotype, a culture of enriched astrocytes was prepared as described above in *Materials and Methods* (Fig. 2a). $CD11b^+$ microglia were purified by magnetic cell sorting and cultured in isolation or together with the enriched astrocytes. Following treatment with GM-CSF and LPS, microglia from both settings were assessed for CD11c and CD40 expression. The low percentage of $CD11b^+$ microglia that expressed CD11c and CD40 on a bed of enriched astrocytes suggests that astrocytes and not other constituents of the mixed glial environment are the primary inhibitors of the microglial DC-like phenotype.

To determine whether soluble molecules were responsible for the repression of the microglia DC-like phenotype, mixed glial cultures were plated onto 6-well plates and isolated microglia onto Transwell™ inserts (0.4 μm pore size) before being treated with GM-CSF and LPS. Microglia thus separated from astrocytes but in contact with soluble factors coming from them failed to mimic the repressed DC-like phenotype typical of co-cultured microglia. Instead, 'transwell' microglia closely resembled their isolated counterparts (Fig. 2b) suggesting that contact with the astrocytic environment is necessary for DC repression. Moreover, such contact was required for the maintenance of surface expression inhibition; removal of the microglia from the astrocytes resulted in increased surface expression of CD40 even when the soluble elements of the astrocytic environment were included (Fig. 2c).

Astrocytes impede the ability of BM-DCs to mature efficiently

To further investigate the ability of astrocytes to maintain immune privilege and regulate DC maturation, we examined the suppressive qualities of an astrocytic environment on the maturation of bone marrow-derived DCs (BM-DCs). These cells are the professional antigen-presenting cells of the immune system and are a vital component of the adaptive immune response. They were cultured with and without astrocytes to determine the effect of astrocytes on the assumption of a mature DC phenotype.

A high percentage of BM-DCs cultured in the absence of astrocytes express CD11c together with co-stimulatory molecules CD40, CD80, and CD86. In contrast, there was a significant decrease in the percentage of cells expressing these markers when BM-DCs were cultured with astrocytes (Fig. 3a and b). Similar to the effect seen with microglia, the

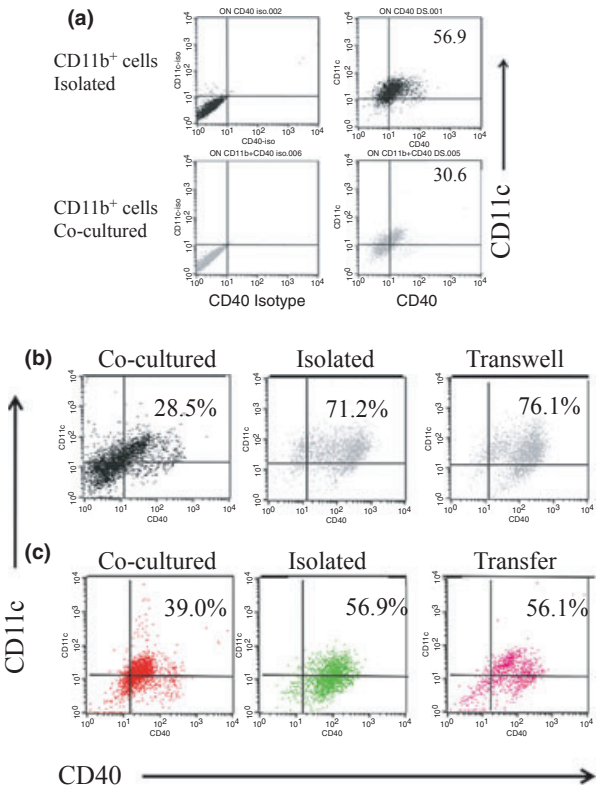

Fig. 2 Purified astrocytes mimic the effect of mixed glia in inhibiting the DC phenotype. (a) Enriched astrocytes were prepared as described in *Materials and Methods*. Microglia were enriched by magnetic cell sorting using $CD11b^+$ beads. They were then cultured with (Co-cultured) or without (Isolated) astrocytes and treated with GM-CSF (25 ng/mL, 5 days) and LPS (50 ng/mL, 2 days). Numbers in the upper right hand quadrants represent the percentage of $CD11c^+CD40^+$ cells. (b) Contact with the astrocytic environment is necessary for astrocytes to modulate microglial DC maturation. B10.A microglia in the presence (Co-cultured) or absence (Isolated) of astrocytes and microglia separated from mixed glial cultures (Transwell) were treated with GM-CSF and LPS as above. Surface expression of CD11c and CD40 were assayed by flow cytometry. Numbers in the upper right hand quadrants represent the percentage of $CD11c^+CD40^+$ cells. Data shown are representative of three experiments performed. (c) Removal of microglia from astrocytes after treatment of GM-CSF (labeled 'Transfer') results in the increased surface expression of CD40. In this experiment, soluble elements from the GM-CSF-treated co-culture were transferred together with the microglia.

percentage of BM-DCs co-expressing CD11c and MHC Class II did not differ significantly between treatments.

Since bone marrow cells are a heterogeneous mix of cells at various stages of maturation, we sought to confirm that the results observed were because of an interaction between BM-DCs and astrocytes alone. To do this, after an initial three-day culture period, $CD11c^+$ cells were isolated from BM using magnetic cell sorting and were plated onto a bed of enriched astrocytes. BM-DCs thus sorted were > 97% pure (Fig. 3d). Purified $CD11c^+$ BM cells were then treated as described

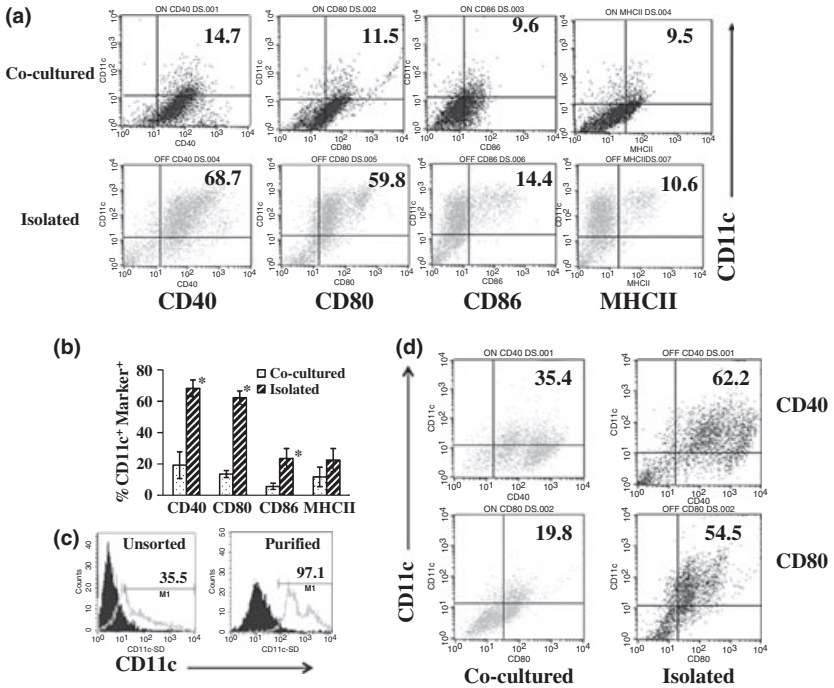

Fig. 3 Astrocytes inhibit the expression of a mature DC phenotype in bone marrow-derived cells. Bone marrow (BM) precursors were obtained from the femurs of B10.A mice and cultured for 3 days in the presence of GM-CSF (15 ng/mL). Floating cells were re-plated and treated with GM-CSF (15 ng/mL) for 5 days and LPS (50 ng/mL) for an additional 2 days in the presence (Co-cultured) or absence (Isolated) of astrocytes. (a) CD11c and co-stimulatory molecule surface expression was determined by flow cytometry. Numbers in the upper right quadrants represent the percentage of $CD11c^+$ cells simultaneously positive for CD40, CD80, CD86, and MHC Class II, respectively. (b) bone marrow-derived DCs (BM-DC) surface expression of co-stimulatory molecules in isolation is significantly higher than those on BM-DCs cultured with astrocytes. Data are expressed as the percentage of cells expressing both CD11c and another co-stimulatory molecule ± SEM over three individual experiments. Data from isolated and co-cultured BM-DCs were compared by a Student's *t*-test. An asterisk indicates $p < 0.05$. (d) A higher percentage of purified $CD11c^+$ cells cultured in the presence of astrocytes show surface expression of CD40 and CD80. Purified $CD11c^+$ BM-DCs (shown in c) were treated with GM-CSF (15 ng/mL) for 5 days and LPS (50 ng/mL) for two more days in the presence (Co-cultured) or absence (Isolated) of astrocytes. Cells were then analyzed for CD11c and CD40 or CD80.

above in the presence or absence of enriched astrocytes and the percentage of cells expressing CD11c and CD40 or CD80 determined by flow cytometry. Similar to data seen with microglia, a significantly higher percentage of isolated BM-DCs were $CD11c^+$ $CD40^+$ or $CD11c^+CD80^+$ compared to BM-DCs in the presence of astrocytes (Fig. 3c). These data suggest that it is astrocytes and neither other bone marrow-derived cells nor other glial components that are responsible for impeding the assumption of the mature DC phenotype.

GM-CSF stimulates mRNA expression in isolated microglia

Because a higher percentage of isolated microglia expressed a mature DC-like phenotype, we sought to examine in more detail the temporal expression of co-stimulatory molecules by examining mRNA during the full course of GM-CSF and LPS treatment. Isolated and co-cultured microglia were treated for various times with GM-CSF. Co-cultured microglia were removed from astrocytes and the cells from both culture conditions examined by QRT-PCR for MHC Class II and the various co-stimulatory molecules.

Isolated microglia responded promptly to GM-CSF treatment with increases in mRNA for co-stimulatory molecules and MHC Class II (Fig. 4a–d). All were elevated by 3 or 6 h after the beginning of treatment. mRNA levels of these molecules in co-cultured microglia lagged behind, rarely attaining the levels seen in the isolated microglia. These data suggest that the microglial response to GM-CSF is inhibited or retarded by the presence of astrocytes.

LPS promotes mRNA and protein expression in co-cultured microglia

A similar time-course following LPS treatment was performed. After a 5-day treatment with GM-CSF, cells were treated for various times with LPS and collected for QRT-PCR analysis. In these experiments, co-cultured microglia, not isolated microglia responded more rapidly and with higher levels of mRNA (Fig. 5a–d). In microglia co-cultured with astrocytes mRNA levels for CD40, CD80, and CD86 increased rapidly, peaking at 3 h and declining thereafter. In contrast, MHC Class II mRNA levels rose slowly but

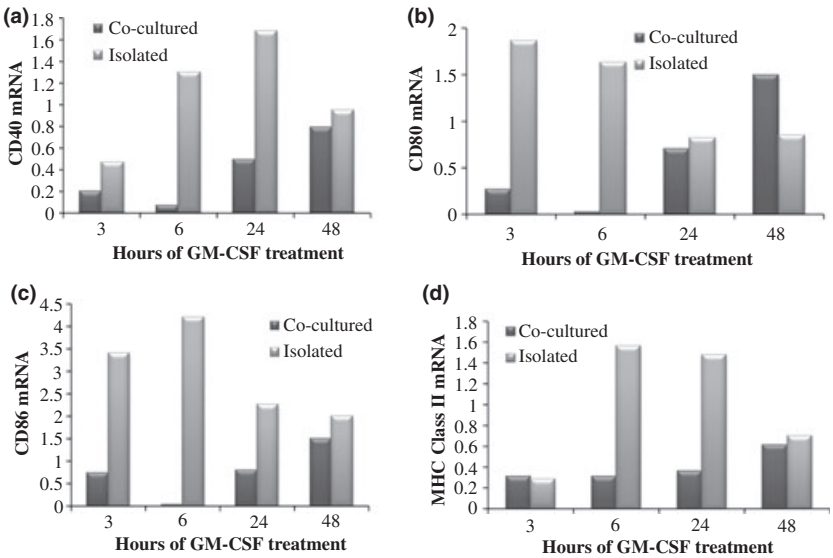

Fig. 4 Microglia were cultured alone (Isolated) or together with astrocytes (Co-cultured) and treated with GM-CSF (25 ng/mL). Microglia were harvested at the various time points indicated after GM-CSF addition and (a) CD40 (b) CD80 (c) CD86 (d) MHC class II mRNA expression assessed using real-time PCR. Data are representative of two experiments performed.

steadily reaching highest levels at 12 h. In the isolated microglia, CD40, CD80, CD86, and MHC Class II expression showed a similar temporal pattern. However, at all time points examined, steady-state mRNA levels in isolated microglia were significantly lower than those of co-cultured microglia. In the case of CD86 and MHC Class II, these levels were strikingly lower.

One possible explanation of such a disparity in mRNA levels would be that isolated microglia had a faster turnover rate for mRNA than their co-cultured counterparts. This was not the case. Measurements of mRNA decay rates were not significantly different in the two culture settings (Fig. 5e–g).

To determine whether CD40 protein surface expression tracked mRNA levels following LPS treatment, we measured its expression and that of CD11c over time using flow cytometry (Fig. 5h–j). This analysis revealed that at the end of a 5-day treatment with GM-CSF (time = 0 h), almost 100% of isolated microglial cells expressed CD40 and CD11c even in advance of LPS treatment (Fig. 5h). Subsequent LPS treatment of the isolated microglia did not increase that percentage further. However, the MFI increased substantially (Fig. 5j). In the isolated microglia, most $CD11c^+$ cells were also positive for CD40. In contrast, microglia in the presence of astrocytes had low percentages of CD40, CD11c, and double-positive cells at the end of GM-CSF treatment, but LPS caused those levels to gradually increase (Fig. 5i). Nevertheless, the percentage of cells expressing CD11c never reached that of isolated cells; therefore, the percentage of cells expressing both markers remained low. In accord with our previous data, at the end of the 48-hr LPS treatment, the percentage of $CD11c^+CD40^+$ cells in isolated microglia was approximately 70%, while the percentage in co-cultured microglia was approximately 50% as indicated on the figure. Unlike their isolated counterparts, co-cultured microglia maintained constant and low MFI levels (Fig. 5j).

Co-cultured microglia produce CD40 protein, but fail to place it onto the cell membrane

Western blots were performed on microglial lysates derived from isolated microglia or co-cultured cells. These revealed significant quantities of protein present in the co-cultured microglia even at times when surface expression was low (Fig. 6a). Thus, if one compares the surface expression of CD40 at the end of GM-CSF treatment ($t = 0$) as seen in Fig. 5i with the protein levels detected by Western blot at the same time point, there is a significant quantity of CD40 protein that is not detected on the cell surface. The HM40-3 antibody revealed three distinct bands of protein all of which appeared to decline somewhat over the 24 h of LPS treatment. In contrast, isolated cells had low levels of protein at the end of GM-CSF treatment with only one band increasing slightly in intensity over the course of LPS treatment. These data suggested that co-cultured microglia produce significant quantities of protein, but that it remains sequestered within the cell until LPS treatment.

To examine this further, cells were prepared for flow cytometry so that surface expression and total protein expression (including intracellular protein) could be compared (Fig. 6b). For these experiments, cells were harvested after a 5-day treatment with GM-CSF or after a subsequent 24-h treatment with LPS. They were then stained as before with antibodies against CD11c and CD40. These cells, then, revealed only surface expression. Half of the cells were then fixed and permeabilized for 30 min and were re-stained with antibodies against CD40. These cells revealed both surface and intracellular protein levels.

Virtually, 100% of both isolated and co-cultured microglia expressed CD40. Differences emerged, however,

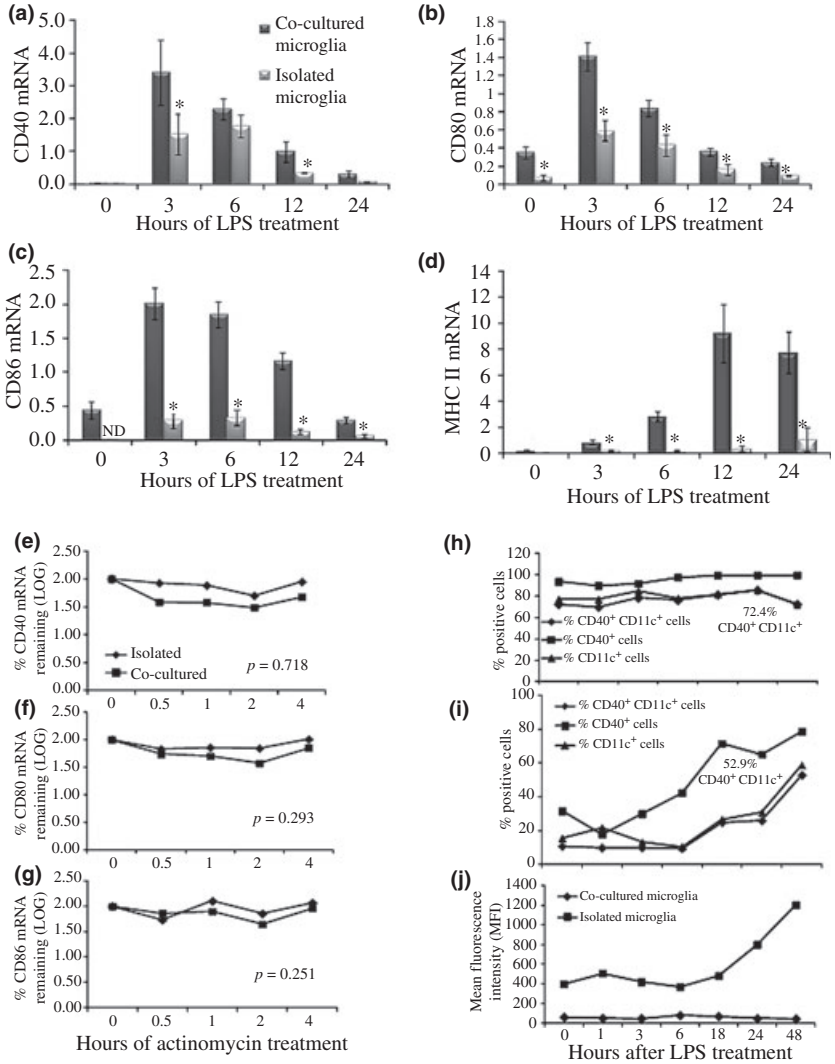

Fig. 5 Microglia were cultured alone (Isolated) or together with astrocytes (Co-cultured) and treated for 5 days with GM-CSF (25 ng/mL). Microglia were harvested at various time points indicated after LPS addition and (a) CD40 (b) CD80 (c) CD86 (d) MHC Class II mRNA expression assessed through real-time PCR. The data are expressed as arbitrary units ± SEM from three separate experiments. Levels at each time point were compared by a Student's *t*-test. The asterisk indicates $p < 0.01$. Data comparisons using a two-way ANOVA yielded identical results. (e–g) mRNA levels are not because of differences in decay rate. Co-cultured and isolated microglia were treated as described in Materials and Methods. At the various time points indicated, cells were harvested and assessed for mRNA by RT-PCR for CD40 (e), CD80 (f), and CD86 (g). *p*-values obtained are noted on the graphs. A repetition of this experiment confirmed that there was no difference in the degradation rate of the mRNA. (h–i) Surface expression of CD11c, CD40 and simultaneous expression of CD11c and CD40 was assessed at various time points following LPS administration in both co-cultured and isolated microglia. Note that maximum expression of CD40 and CD11c in the isolated cells has already been achieved following GM-CSF stimulation. Data are representative of three experiments performed. (j) mean fluorescence intensity (MFI) of CD40 in isolated and co-cultured microglia. While not increasing the percentage of cells expressing CD40, isolated cells increase MFI with LPS treatment.

when only surface expression was analyzed. Following GM-CSF treatment only about 35% of the co-cultured cells showed surface expression, while more than 90% had reached the cell surface in the isolated microglia. Moreover, the MFI of the protein in the isolated cells was substantially higher than their co-cultured sisters. Following 24 h of LPS treatment, co-cultured cells increased the percentage of cells expressing surface CD40 to approximately 70%, but the MFI remained low. These data are in accord with those in Fig. 5 and suggest that much of the protein translated in the co-cultured cells is sequestered intracellularly.

Analysis by ImageJ® software of the cellular distribution of CD40 shows that isolated microglia express CD40 primarily at the cell's edges while co-cultured microglia show distribution surrounding a central nucleus (Fig. 7).

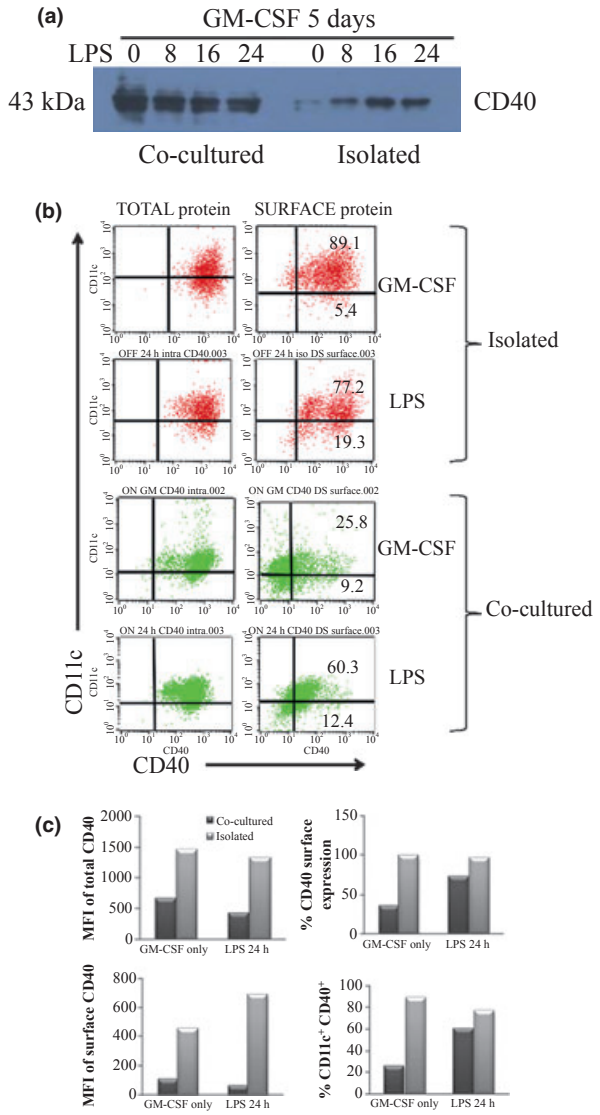

Fig. 6 Surface vs. intracellular expression of CD40. (a) Western blot analysis of CD40 at various time points following LPS stimulation. Total cell lysates were prepared for western blot analysis using the HM40-3 anti-CD40 antibody. Total CD40 protein was substantially higher and showed multiple isoforms at all time points in the co-cultured microglia even though flow cytometry indicates lower surface expression (Fig. 1). Western blots have been performed three times with identical results. (b) Isolated and co-cultured microglia were prepared for flow cytometry both following GM-CSF treatment and also after a 24-h LPS treatment. Cells were stained with and without a permeabilizing agent to determine TOTAL CD40 protein versus SURFACE protein. A representative experiment of 3 performed is shown. (c) Data from (b) are depicted in graphic form.

Immunocytochemistry further revealed that most of the CD40 is harbored within the ER (Fig. 8), not in lysosomes or early endosomes. Even after 24 h of treatment with LPS, CD40 largely co-localizes with the ER in co-cultured microglia, while isolated microglia maintain a robust surface expression. These data suggest that astrocytes retard the placement of CD40 onto the cell surface both during GM-CSF treatment and even following LPS administration.

Discussion

As microglia are most often studied in isolation, the modulatory role of astrocytes on microglial function has not been extensively examined. The few studies that have been done show that astrocytes render microglia resistant to activation, showing decreased production of inflammatory molecules including pro-inflammatory cytokines, nitric oxide, and other superoxide anions (Aloisi *et al.* 1997; Tichauer *et al.* 2007). The ability of astrocytes to down-regulate the antigen-presenting function of invading monocytes has been reported (Hailer *et al.* 1998), and recent bioinformatics studies have highlighted the importance of the microenvironment in regulating microglial expression of various molecules (Schmid *et al.* 2009).

We have examined the ability of microglia to assume a mature DC-like phenotype in the presence and absence of astrocytes, and find that microglia in a complex glial environment behave quite differently from their isolated sisters. Surface expression of co-stimulatory molecules necessary for efficient antigen presentation is far more robust on isolated microglia than that seen on microglia in a mixed glial environment. Consistent with the data of others (Hailer *et al.* 1998), astrocytes also restrained the maturation of BM-DC precursors. This would suggest that under normal conditions maturation of BM-DC precursors that find their way into the brain is discouraged.

Our data thus suggest that the brain parenchyma – and particularly the astrocytic environment – presents an environment inimical to the development of a fully mature DC-like phenotype even in the face of a potent bacterial stimulus. These data are consistent with studies *in vivo* that have shown that DCs extracted from brains of adult mice displaying features of multiple sclerosis express an immature DC phenotype (Suter *et al.* 2003) thereby playing an apparent regulatory role. Thus, although isolated microglia have been shown time and time again to express the pro-inflammatory M1 phenotype, their presence in a more complex cellular environment substantially modifies that response. The maintenance of a more quiescent M2 phenotype seems largely to be the responsibility of astrocytes. While others have identified a variety of soluble molecules that dampen microglial assumption of a DC-like phenotype (notably TGFβ, IL-10, and IL-4 (Frei *et al.* 1994; Kim *et al.* 2002; Ledeboer *et al.* 2000; Ponomarev *et al.* 2007), our data suggest that non-soluble elements of the astrocytic environment are also potent inhibitors.

Investigation of mRNA coding for co-stimulatory molecule expression after LPS treatment revealed that while surface expression of co-stimulatory molecules on co-

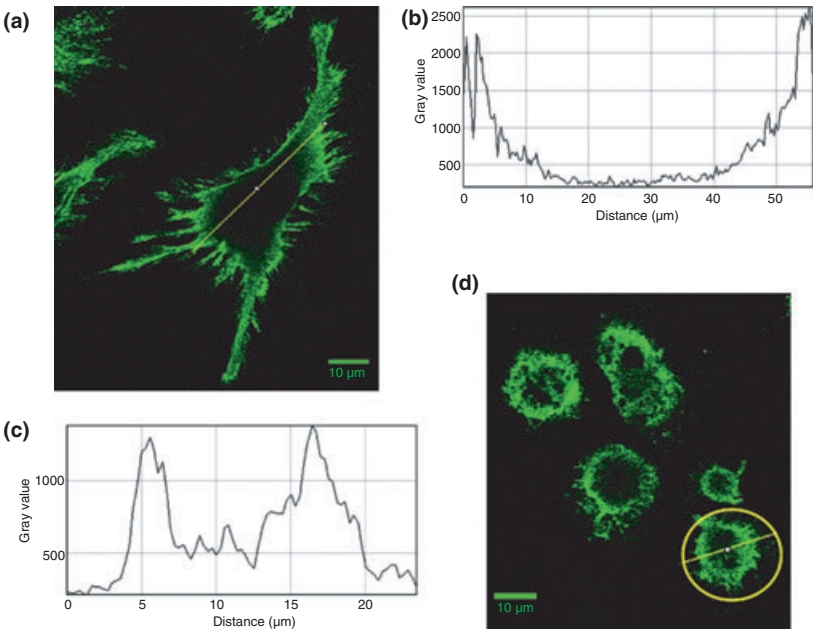

Fig. 7 CD40 immunoreactivity in isolated (a) and co-cultured (d) microglia reveal variability in cellular localization 24 h after LPS treatment. Histogram analysis by ImageJ® software reveals that isolated microglia (b) express CD40 at the cell surface, while co-cultured microglia retain it intracellularly clustered primarily around the cell nucleus (c). The extent of the cell boundary in the co-cultured microglial cell is indicated by a circle (d).

cultured microglia was low, mRNA levels were high. We considered the possibility that the lower surface expression might be the result of suppression of translation by microRNAs. Indeed, a recent study has identified miRNA-124 as being a key regulator of microglial quiescence (Ponomarev *et al.* 2011). In our experiments, co-cultured microglial expression of miRNA-155, miRNA146a, and miRNA146b – microRNAs implicated in DC expression and autoimmunity (Baltimore *et al.* 2008; O'Connell *et al.* 2010; Boldin *et al.* 2011) – were substantially elevated over their isolated counterparts (data not shown), but Western blots suggested that inhibition of CD40 translation was not the underlying problem.

We further examined co-stimulatory molecule expression during the GM-CSF treatment that preceded LPS. Co-cultured microglia responded to GM-CSF – they proliferated and gradually up-regulated CD40 and CD80 mRNA – but failed in placing translated protein onto the cell membrane. Instead, they awaited signals from LPS to up-regulate co-stimulatory molecules further and move translated protein onto the cell surface. Various signaling pathways for GM-CSF subserve proliferation, survival, and differentiation. Jak3 is required for GM-CSF-induced differentiation of myeloid DCs (Rane *et al.* 2002; Mangan *et al.* 2006). Thus, the down-regulation of GM-CSF-induced Cdk2, Cdk4, Cdk6, and Cyclin E together with the concomitant up-regulation of the Jak3pathway would shift cells from a proliferative phenotype (as seen with co-cultured cells) to one capable of differentiation (as seen with isolated cells). We hypothesize, therefore, that access to the Jak3 pathway is suppressed by astrocytes via a contact-mediated mechanism that prevents GM-CSF treatment from effecting maturation in co-cultured microglia.

After LPS treatment, even co-cultured microglia up-regulate mRNA levels, but the percentage of cells expressing surface CD11c never attains levels comparable to those seen in the isolated cells. Moreover, co-cultured cells do not increase the MFI of CD40 expression even as the percentage of expressing cells increases. These data taken together suggest that astrocytes do not exert a global restriction on microglial transcription or translation, but that other processes are impacted. Western blots and immunocytochemistry confirm that co-cultured microglia produce substantial quantities of CD40, but that it is sequestered within the ER following GM-CSF treatment and for at least 24 h after LPS treatment. Thus, it appears that trafficking processes that export protein from the ER and onto the cell surface are among the processes that are stalled. Alternatively, retrieval systems that facilitate movement of proteins from the Golgi to the ER may be active.

The necessity of contact with the astrocytic environment to quash DC maturity is consistent with data of others who have found that laminin and fibronectin alter microglial differentiation (Chamak and Mallat 1991) and promote more immature DC-like phenotypes (Brand *et al.* 1998; Garcia-Nieto *et al.* 2010). The interplay between the extracellular matrix (ECM) and DCs has been noted by others (Brand *et al.* 1998; Jancic *et al.* 1998; Kohl *et al.* 2007; Sprague *et al.* 2011). In the case of microglia, we have observed that isolated microglia cultured on a complex ECM mimic (Matrigel™) behave more like co-cultured microglia (data not shown). In short, the interplay between

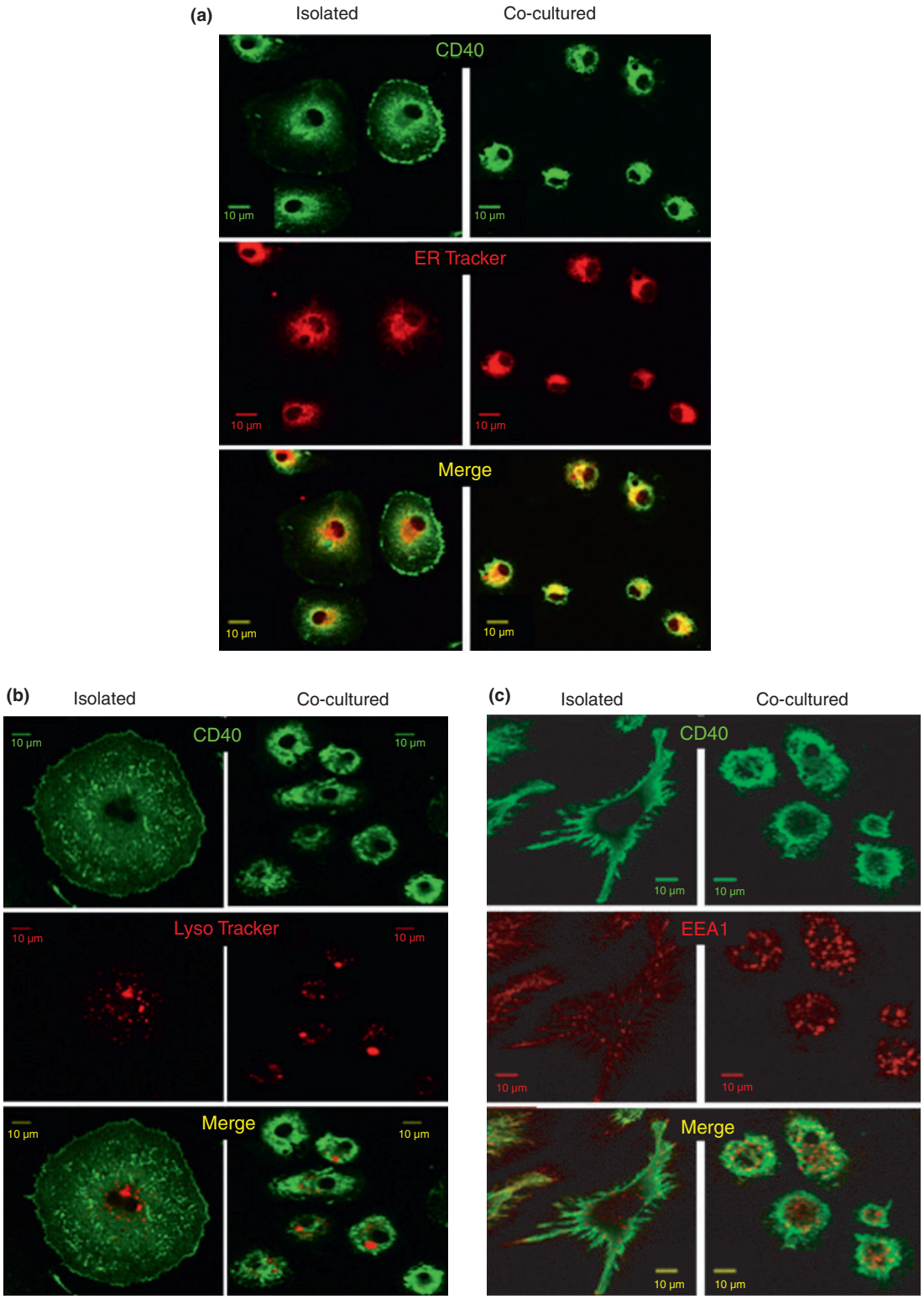

Fig. 8 Immunocytochemistry reveals CD40 sequestered in co-cultured cells in the ER. Isolated and co-cultured microglia were prepared as described in the text. After 24 h of LPS treatment, cells were prepared for simultaneous expression of CD40 and ER using ER Tracker™ (a), CD40 and lysosomes using LysoTracker™ (b) or CD40 and early endosomes using an antibody against EEA1 (c). Only ER Tracker™ showed substantial overlap with CD40 expression in co-cultured microglia.

cells and their ECM environment is crucial to their function.

This is particularly important when investigating 'tumor tolerance' in the CNS. Tumors are notoriously adept at suppressing or evading anti-tumor immunity by encouraging macrophage polarization to the M2 phenotype (Sica *et al.* 2006; Soares-Schanoski *et al.* 2012) or suppressing or promoting defective differentiation of monocytic-derived DCs (Almand *et al.* 2000; Wang *et al.* 2006; Liu *et al.* 2009). Glioblastomas dramatically inhibit monocytic assumption of a mature DC phenotype possibly by blocking positive signaling pathways (Kostianovsky *et al.* 2008). Our data would suggest that investigating cell-to-cell interactions with the tumor cell environment that might trigger these alternative pathways would be highly beneficial.

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The authors have no conflicts of interest to declare.

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